Distinct peptide signals in the UmuD and UmuD′ subunits of UmuD/D′ mediate tethering and substrate processing by the ClpXP protease

Saskia B. Neher*, Robert T. Sauer*, and Tania A. Baker*†‡

*Department of Biology and †Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA 02139

Contributed by Robert T. Sauer, September 11, 2003

The *Escherichia coli* UmuD′ protein is a component of DNA polymerase V, an error-prone polymerase that carries out translesion synthesis on damaged DNA templates. The intracellular concentration of UmuD′ is strictly controlled by regulated transcription, by posttranslational processing of UmuD to UmuD′, and by ClpXP degradation. UmuD′ is a substrate for the ClpXP protease but must form a heterodimer with its unabbreviated precursor, UmuD, for efficient degradation to occur. Here, we show that UmuD functions as a UmuD′ delivery protein for ClpXP. UmuD can also deliver a UmuD partner for degradation. UmuD resembles SspB, a well-characterized substrate-delivery protein for ClpX, in that both proteins use related peptide motifs to bind to the N-terminal domain of ClpX, thereby tethering substrate complexes to ClpXP. The combined use of a weak substrate recognition signal and a delivery factor that tethers the substrate to the protease allows regulated proteolysis of UmuD/D′ in the cell. Dual recognition strategies of this type may be a relatively common feature of intracellular protein turnover.

Regulation of protein levels by proteolysis is an integral part of stress responses in all cells. In *Escherichia coli*, for example, the ClpXP protease degrades transcription factors that control responses to starvation and DNA damage, as well as specific proteins induced by these stresses (1, 2). ClpXP is composed of a ATP-dependent protein unfoldase, the $ClpX_6$ hexamer, and a double-ring serine protease, $ClpP_{14}$ (3, 4). ClpX selects substrates for degradation, unfolds them, and translocates the unfolded polypeptide into a chamber within ClpP, where degradation occurs (5–7). Importantly, ClpXP degrades different substrate proteins at different times, depending on growth or environmental conditions. Therefore, it is critical to understand the mechanisms that permit the proper substrates to be selected for degradation in a regulated and coordinated fashion.

One fundamental mode of substrate recognition involves the binding of a substrate-processing site on ClpX to a peptide degradation signal, which is often at or near the N or C terminus of the target protein (8–10). Peptide degradation sequences may be constitutively recognized or become accessible to ClpX only after cleavage by another protease or after a conformational change (11, 12). After recognition of the peptide degradation signal by the ClpX processing site, ATP-dependent conformational changes in ClpX are thought to generate a transient "pulling" force that destabilizes the attached native protein (13). By using repeated cycles of ATP hydrolysis, ClpX unfolds the protein substrate and translocates it into ClpP for degradation. Some peptide degradation signals are sufficient to cause virtually any attached protein to be efficiently proteolyzed by ClpXP. For example, addition of the ssrA tag [a peptide added cotranslationally to nascent polypeptides when bacterial ribosomes stall (14, 15)] will target even hyperstable proteins for ClpXP degradation (5, 6, 13, 16).

A second mode of substrate recognition by ClpX involves tethering sites that interact with substrate-delivery or adaptor proteins. These accessory molecules enhance the degradation of specific ClpXP substrates without themselves being degraded. For example, the response regulator RssB forms a complex with the starvation sigma factor, σ^S, and accelerates its degradation by ClpXP (17). Likewise, SspB binds specifically to ssrA-tagged proteins, helping deliver them to ClpXP for degradation (18). Although σ^S and ssrA-tagged proteins carry ClpX-degradation signals (10, 19), RssB and SspB improve the efficiency of their degradation at low substrate concentrations by tethering them to ClpXP (17, 18, 20). This mechanism has been most clearly demonstrated for SspB-mediated degradation of ssrA-tagged proteins. One part of the SspB protein binds to the ssrA-degradation tag whereas another part interacts with a tethering site on ClpX (20). When ClpX, SspB, and an ssrA-tagged substrate are all present, a stable ternary delivery complex is efficiently formed at concentrations lower than those that would support stable binding of ClpX directly to the ssrA-tagged protein (21).

The DNA damage-inducible UmuD′ protein is an important ClpXP substrate *in vivo*. An essential subunit of the error-prone translesion DNA polymerase (polV), UmuD′ is synthesized as a precursor, UmuD. After DNA damage, UmuD cleaves itself between residues 24 and 25 in a RecA-mediated reaction to generate UmuD′ (22, 23). Both UmuD and UmuD′ form homodimers, but UmuD/D′ heterodimers form preferentially (24). Importantly, UmuD′ seems to be degraded by ClpXP only when it is bound to UmuD (25). Although residues within the precursor region of UmuD (and thus unique to UmuD) are essential for UmuD′ degradation in UmuD/D′ heterodimers, the UmuD subunit is not degraded. Moreover, homodimers of UmuD have been reported to be resistant to ClpXP degradation (25). Hence, in this trans-targeting reaction, the UmuD subunit of a UmuD/D′ heterodimer seems to provide sequence information essential for the ClpXP degradation of the UmuD′ subunit even though neither subunit seems to be degraded on its own.

Here, we show that ClpXP degradation of the UmuD′ subunit of a UmuD/D′ heterodimer occurs in a manner similar to SspB-mediated degradation of ssrA-tagged substrates. A peptide motif in the precursor region of UmuD resembles a motif used by SspB to tether itself to the N-terminal domain of ClpX (20). This peptide sequence in UmuD has previously been shown to be important for degradation of UmuD′ (25). We show that UmuD-dependent degradation of UmuD′ by ClpXP can be blocked by the SspB-tethering peptide and that the SspB-tethering motif can replace the sequence in UmuD. Furthermore, we demonstrate that the N-terminal domain of ClpX, which mediates interactions with SspB (refs. 26 and 27; S. Siddiqui, I. Levchenko, D. Wah, and G. Hersch, personal communication), is also essential for efficient UmuD-dependent degradation of UmuD′. Thus, UmuD behaves like a ClpX

Abbreviation: XB, ClpX-binding.

‡To whom correspondence should be addressed. E-mail: tabaker@mit.edu.

delivery factor; it carries a peptide motif essential for tethering itself and its dimeric partner to ClpX. In fact, we find that UmuD can also deliver another UmuD subunit for ClpXP degradation. Additional peptide signals recognized by ClpX are present in the UmuD′ protein sequence, at least one of which seems to function as a primary degradation signal. The joint use of tethering peptides and low-affinity primary degradation signals permits combinatorial control in regulated protein turnover.

Materials and Methods

Proteins and Peptides. Purifications for ClpP (5) and Arc (28) used established procedures. $ClpX^{\Delta 1-46}$ was a gift from S. Siddiqui (Department of Biology, Massachusetts Institute of Technology). *E. coli* BL21 transformed with pAG99 or pAG98 (29) was used for the purification of $UmuD_2$ (30) or $UmuD'_2$ (31). To generate ^{35}S-labeled UmuD and UmuD′, cells were grown in M9 minimal media lacking methionine to an OD_{600} of 0.4 and induced with 0.4 mM isopropyl β-D-thiogalactoside for 80 min. Express ^{35}S protein-labeling mix (NEN) was added to 20 μCi/ml (1 Ci = 37 GBq) of culture, and cells were grown for an additional 30 min before harvesting. ^{35}S-labeled UmuD and UmuD′ were then purified by the procedures for unlabeled proteins. Plasmids for the expression of $UmuD^{R37A}$, $UmuD^{XB}$, and $UmuD'^{R37A}$ were generated from pAG98 and pAG99 by using the Stratagene QuikChange kit, and the mutant proteins were purified like their wild-type counterparts. The SspB ClpX-binding (XB) peptide had the sequence NH_2-CRGGRPALRVVK-COOH (20). A UmuD peptide with the sequence NH_2-WKPADLREIVT-COOH was synthesized for inhibition studies.

ClpX was purified from 10 liters of WM53/pTB9 cells grown at 37°C in 25.5 g/liter Bacto tryptone, 15.5 g/liter yeast extract, 4 g/liter NaCl, and 100 mg/liter ampicillin in a BioFlo IV fermenter (New Brunswick Scientific) to an OD_{600} of 8, shifted to 25°C, and induced with 0.25 mM isopropyl β-D-thiogalactoside. After 3 h, cells were harvested and resuspended in 4 ml buffer A (50 mM Tris·HCL, pH 8.2 at 4°C/100 mM KCl/1 mM $MgCl_2$/5 mM DTT/10% glycerol) per gram of cell paste. Set III protease inhibitors (Calbiochem) were added to 0.17 μl/ml suspension. After lysis by French press at 10,000 psi, insoluble material was removed by centrifugation, $AmSO_4$ was added to 35% saturation, and precipitated material was collected and dissolved in buffer A to 10 mg/ml. The conductivity was matched to that of buffer PS_A (50 mM Tris·HCL, pH 8.2 at 4°C/0.5 M $AmSO_4$/0.5 mM DTT/10% glycerol), and the protein concentration was adjusted to 5 mg/ml. After centrifugation, the supernatant was loaded onto a phenyl Sepharose HR column (Amersham Pharmacia) at 3–4 mg of protein per ml of resin. ClpX eluted ≈80% through a linear gradient to buffer A and was precipitated with 35% $AmSO_4$, redissolved, and desalted into buffer A by using a HiPrep 26/10 column (Amersham Pharmacia). Protein was loaded onto Q-Sepharose (3 mg of protein per ml of resin) and eluted with a gradient to buffer A plus 300 mM KCl. Peak fractions containing ClpX were loaded onto a Bio-gel HTP hydroxyapatite (Bio-Rad) column (4 mg of protein per ml of resin) and eluted with a linear gradient to 260 mM K_2HPO_4/KH_2PO_4 (pH 7.2), 5 mM DTT, and 10% glycerol. Peak fractions were pooled, precipitated with 35% $AmSO_4$, and redissolved and desalted into buffer A plus 20 mM $AmSO_4$ for storage.

Degradation Assays. Buffer NB (50 mM Tris·Cl, pH 8.0/100 mM KCl/10 mM $MgCl_2$/1 mM DTT) was used for ClpXP degradation of UmuD/D′, $UmuD_2$, and $UmuD'_2$. PD buffer (5) was used for Arc-ssrA degradation. An ATP regeneration system (16 mM creatine phosphate/0.32 mg/ml creatine kinase/5 mM ATP) was included in all ClpXP degradation reactions. Degradation reactions were preformed at 30°C and contained 0.3 μM $ClpX_6$, 0.8 μM $ClpP_{14}$, and the indicated concentration of substrate. When monitoring the release of acid-soluble peptides, reactions were stopped by adding trichloroacetic acid to 10%, samples were placed on ice for 20 min, and insoluble material was removed by centrifugation at 4°C in a microcentrifuge (14,000 × *g*). Radioactivity in the supernatant was assayed by scintillation counting. Proteolysis of UmuD and UmuD′ is reported as the number of picomoles degraded in a reaction volume of 2.4 μl. For degradation monitored by SDS/PAGE, reactions were stopped by adding SDS sample buffer and freezing in liquid nitrogen. Samples were electrophoresed on 15% polyacrylamide gels, stained by using SYPRO orange (Molecular Probes), and visualized by using a Molecular Dynamics model 595 FluorImager.

For identification of UmuD degradation products by mass spectrometry, $UmuD_2$ (10 μM) was digested with ClpXP for 2 h at 30°C. The resulting peptides were separated by reverse-phase chromatography on a Vydac (Hesperia, CA) C18 Mass Spec HPLC column by using a 1-h gradient from 5% to 95% buffer B (buffer A is 5% acetonitrile and 0.1% formic acid; buffer B is 90% acetonitrile, 0.1% formic acid, and 10% isopropanol). Peptides were identified on an LCQ electrospray ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA). Sequence analysis was achieved by collision-induced fragmentation within the ion trap; peptides reported had a Sequest cross-correlation value of 2.5 or higher.

Peptide Array. A peptide array containing UmuD peptide sequences was prepared by the MIT Biopolymers facility by using an Abimed instrument (Abimed Analysentechnik, Langenfeld, Germany). Each UmuD peptide sequence contained 12 residues and was offset by 2 residues from the succeeding peptide. Peptides interacting with ClpX were detected by indirect Western blotting using an anti-ClpX antibody as described (9), and the intensity of the interaction was quantified by using IMAGEQUANT (Molecular Dynamics).

Results

UmuD Binds ClpX in a Manner Similar to SspB. We initially noticed that a sequence near the N terminus of UmuD ($L^9R^{10}E^{11}I^{12}$), which had been implicated previously in mediating interactions between UmuD and ClpX (25), resembled a peptide motif near the C terminus of SspB ($L^{161}R^{162}V^{163}V^{164}$) that tethers this delivery factor to ClpX (20). A peptide (XB) carrying this SspB sequence binds ClpX and inhibits SspB-stimulated degradation (20). Moreover, variants of SspB with L161A or V164A mutations are defective in substrate delivery (20).

We investigated the significance of the similarity between the UmuD and SspB peptide sequences by testing the effect of the XB peptide on UmuD-supported ClpXP degradation of UmuD′. The XB peptide inhibited ClpXP degradation of ^{35}S-labeled UmuD′ in UmuD/D′ heterodimers (Fig. 1*A*), with half-maximal inhibition at an XB peptide concentration of ≈50 μM. This inhibition was specific because high concentrations of the XB peptide did not inhibit ClpXP degradation of Arc-ssrA, a substrate unrelated to UmuD/D′ (Fig. 1*A*). These data support a model in which the XB peptide competes with UmuD for binding to ClpX, thereby inhibiting UmuD′ degradation. We also found that a UmuD peptide carrying the *LREI* motif inhibited UmuD-dependent degradation of UmuD′, although ≈10-fold less efficiently than the SspB XB peptide (data not shown).

Previous experiments have shown that changing the *LREI* sequence of UmuD to *AAAA* results in a variant that is ineffective in supporting degradation of UmuD′ (25). We reasoned that replacing the UmuD sequence with the SspB sequence might improve the ability of UmuD to support ClpXP degradation of UmuD′ in heterodimers. This outcome was observed (Fig. 1*B*). A UmuD mutant ($UmuD^{XB}$) with $E^{11}I^{12}V^{13}$ replaced by $V^{11}V^{12}K^{13}$ (resulting in the same *LRVVK* sequence found at the C terminus of *E. coli* SspB) supported ClpXP degradation of

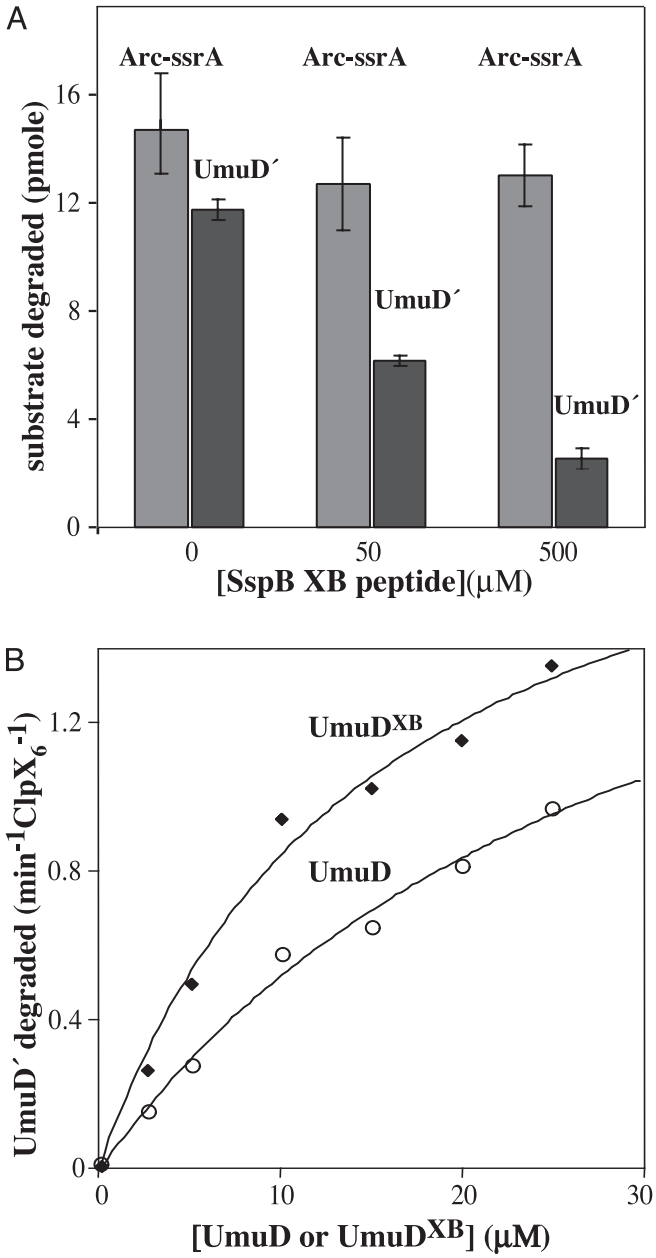


Fig. 1. (*A*) ClpXP degradation of ^{35}S-labeled UmuD′ in UmuD/D′ heterodimers (10 μM) or ^{35}S-labeled Arc-ssrA (10 μM) was measured after 30 min (for UmuD′) or 2 min (for Arc-ssrA) in the presence of 0, 50, or 500 μM SspB XB peptide. (*B*) ClpXP degradation of ^{35}S-labeled UmuD′ (10 μM) was measured as a function of the concentration of UmuD (K_M = 31.8 ± 8.9 μM; V_{max} = 2.1 ± 0.3 min^{-1}·ClpX$_6^{-1}$) or UmuD^{XB} (K_M = 15.4 ± 3.4 μM; V_{max} = 2.1 ± 0.4 min^{-1}·ClpX$_6^{-1}$). Steady-state kinetic parameters were obtained by fits to the Michaelis–Menten equation.

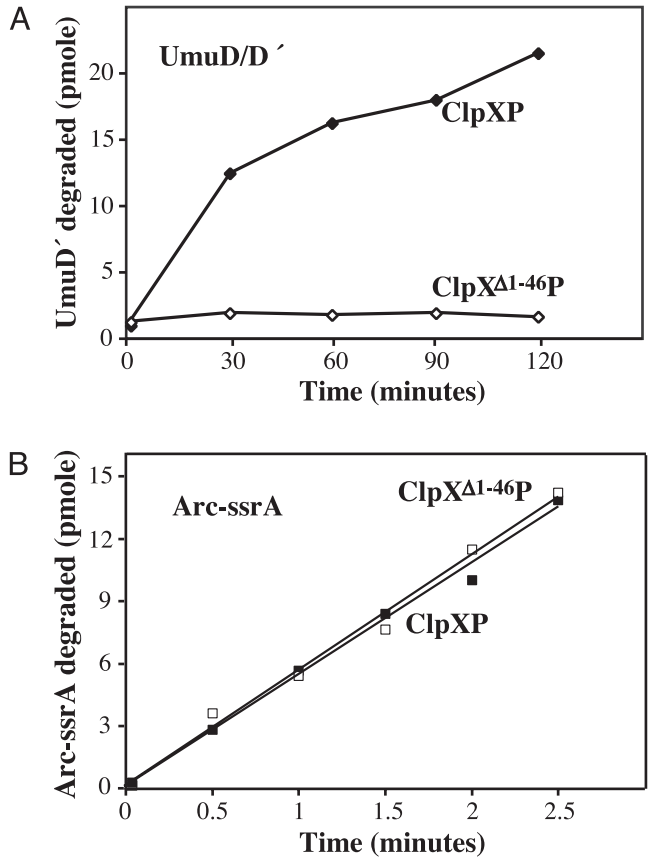
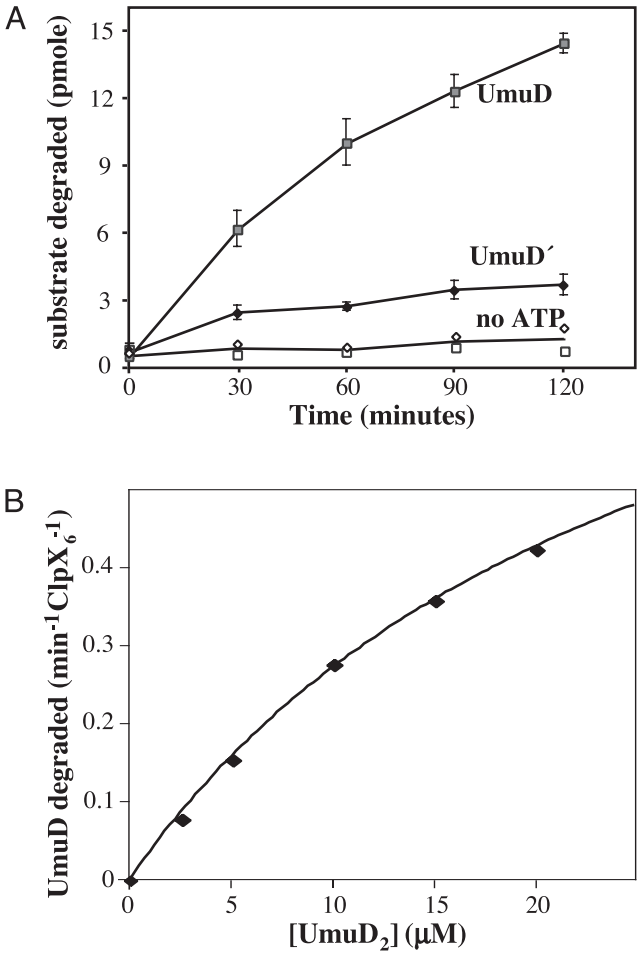


Fig. 2. Degradation of ^{35}S-labeled UmuD′ in UmuD/D′ heterodimers (*A*) or Arc-ssrA (*B*) by ClpXP and ClpX$^{\Delta 1-46}$P. In all experiments, ClpX$_6$ or ClpX$^{\Delta 1-46}$ was present at 0.3 μM, ClpP$_{14}$ was present at 0.8 μM, and substrates were present at 10 μM.

UmuD′. Importantly, UmuD^{XB} ($K_M \approx 15$ μM) was more effective than UmuD ($K_M \approx 32$ μM) at promoting ClpXP degradation of UmuD′ (Fig. 1*B*). These data strongly suggest that the XB region from SspB and the *LREI* motif from UmuD serve equivalent functions in ClpXP-mediated degradation.

We next investigated whether the function of UmuD in degradation of UmuD′ was compromised when the N-terminal domain of ClpX was deleted in the ClpX$^{\Delta 1-46}$ variant. The N-terminal domain of ClpX binds the XB peptide of SspB (D. Wah, G. Hersch, and I. Levchenko, personal communication) and is essential for SspB stimulation but not for degradation of ssrA-tagged substrates (refs. 26 and 27; S. Siddiqui, personal communication). UmuD-supported degradation of UmuD′ by ClpX$^{\Delta 1-46}$P was severely diminished (Fig. 2*A*). After a 2-hour incubation of UmuD/D′ with ClpX$^{\Delta 1-46}$P, only minimal degradation of UmuD′ was observed under conditions where degradation by wild-type ClpXP was robust. As expected, Arc-ssrA was degraded efficiently both by ClpX$^{\Delta 1-46}$P and ClpXP (Fig. 2*B*). These results show that UmuD-dependent delivery of UmuD′ to ClpXP requires the first 46 aa of ClpX.

UmuD Is also a ClpXP Substrate. Because UmuD has all of the sequence information present in UmuD′ but has been reported to be resistant to ClpXP degradation (25), we were interested in potential mechanisms by which ClpXP might discriminate between these proteins. However, control experiments indicated that ^{35}S-labeled UmuD$_2$ homodimers were degraded by ClpXP *in vitro* (Fig. 3*A*), in a reaction dependent upon ClpX and ATP (Fig. 3*A*; data not shown). Indeed, the steady-state kinetic parameters for ClpXP degradation of UmuD$_2$ ($K_M \approx 26$ μM; $V_{max} \approx 1$ min^{-1}·ClpX$_6^{-1}$) indicate that UmuD$_2$ homodimers are degraded with an efficiency similar to the UmuD′ subunit of the UmuD/D′ heterodimer (Figs. 1*B* and 3*B*). We considered that the apparent degradation of UmuD$_2$ by ClpXP might actually result from degradation of UmuD/D′ molecules generated by autocleavage during the reaction. However, MS/MS analysis of the fragments resulting from ClpXP degradation of UmuD$_2$ revealed peptides overlapping the Cys–Gly peptide bond where UmuD is cleaved to generate UmuD′ (Fig. 3*C*). This result shows that unprocessed UmuD is a substrate for ClpXP degradation.



C UmuD´ Cleavage Site

NH_2...LVQCGFPSPAADYVEQRIDLNQLL...COOH

CGFPSPAADYVEQRIDLN
CGFPSPAADYVEQRIDL
CGFPSPAADYVEQRIDLNQ

ClpXP-generated peptides

Fig. 3. (*A*) ClpXP degradation of 10 μM ^{35}S-labeled $UmuD_2$ or $UmuD'_2$. (*B*) Michaelis–Menten plot of ClpXP-mediated degradation of increasing concentrations of $UmuD_2$ (K_M = 26.4 ± 2.3 μM; V_{max} = 1.2 ± 0.1 min^{-1}·$ClpX_6^{-1}$). (*C*) Sequences of peptides that overlap the site of autocleavage between Cys-24 and Gly-25 were identified by tandem mass spectrometry after ClpXP-mediated degradation of $UmuD_2$.

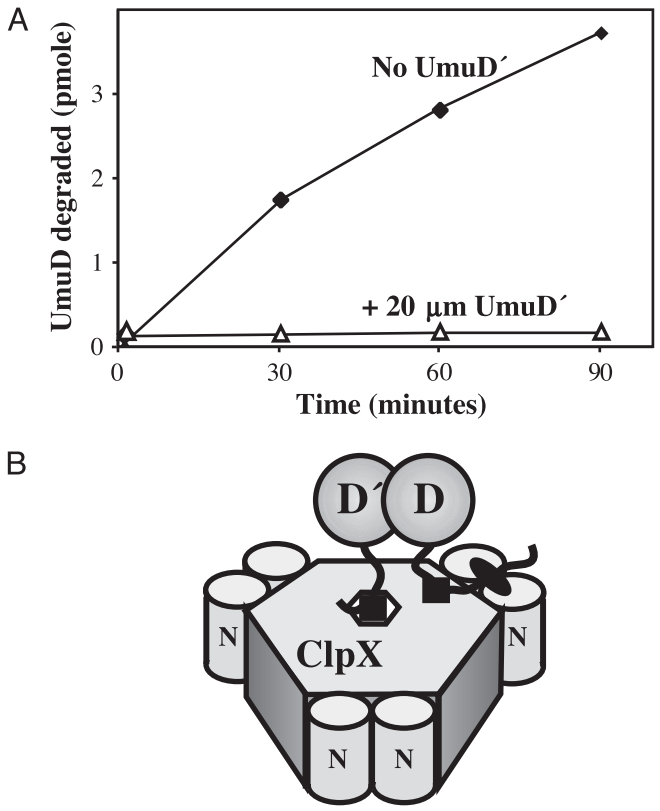


Fig. 4. (*A*) ClpXP degradation of ^{35}S-labeled UmuD (5 μM) is inhibited by increasing concentrations of UmuD′. (*B*) Schematic representation of trans-targeting. A tethering motif (shown as an oval) on the UmuD subunit of the UmuD/D′ heterodimer binds to the N-terminal domain of ClpX, thereby leashing its UmuD′ partner to the enzyme and allowing a weak degradation tag (shown as a square) to interact with the central protein-processing pore.

Two-Site Model for ClpXP Interaction. Because UmuD in a $UmuD_2$ homodimer can be degraded by ClpXP, we revisited the question of ClpXP sensitivity of UmuD in a UmuD/D′ heterodimer. Previous work (25) established that the UmuD subunit of the heterodimer is not degraded and that UmuD can in fact catalytically target excess UmuD′ for ClpXP degradation. In agreement with these studies, we found that a 4-fold excess of unlabeled UmuD′ almost completely inhibited ClpXP degradation of ^{35}S-labeled UmuD (Fig. 4*A*). Because $UmuD'_2$ homodimers are poor substrates for ClpXP and the unlabeled UmuD′ was efficiently degraded in this experiment (data not shown), the most likely mechanism of inhibition is that the addition of UmuD′ leads to a decrease in the population of $UmuD_2$ homodimers as UmuD/D′ heterodimers are formed. Thus, as expected from previous studies (25), we conclude that only the UmuD′ subunit in a UmuD/D′ heterodimer is degraded; this degradation releases the UmuD subunit to form dimers with a new UmuD′ partner.

To explain why UmuD is degraded when present as a homodimer, but only UmuD′ is degraded within the heterodimer, we propose the following two-site recognition model. When ClpXP recognizes a UmuD/D′ heterodimer or a $UmuD_2$ homodimer, only one of the two subunits can be degraded efficiently because one subunit interacts with a "tethering" site on ClpX whereas the second subunit is presented to the "substrate processing" site on ClpX. By this model, UmuD′ would be the only subunit degraded in a UmuD/D′ heterodimer because it lacks the sequence motif required to interact with the tethering site on ClpX. A schematic representation of this model is shown in Fig. 4*B*. In a UmuD homodimer, by contrast, either subunit could bind to the tethering site or to the substrate-processing site, and thus either subunit could be a substrate. However, for each round of binding of the homodimer to ClpXP, only the subunit bound to the "substrate processing" site will be degraded (see *Discussion*).

Sequence Information in UmuD′ Contributes to Its Recognition by ClpXP. We also tested ClpXP degradation of ^{35}S-labeled $UmuD'_2$ homodimers and found that they were degraded by ClpXP, although slowly compared with $UmuD_2$ homodimers or UmuD′ in a UmuD/D′ heterodimer. Only a small fraction of the UmuD′ homodimer was converted to acid-soluble peptides in a 2-h incubation (Fig. 3*A*). However, this low level of degradation was consistently higher than that detected in reactions lacking ClpX

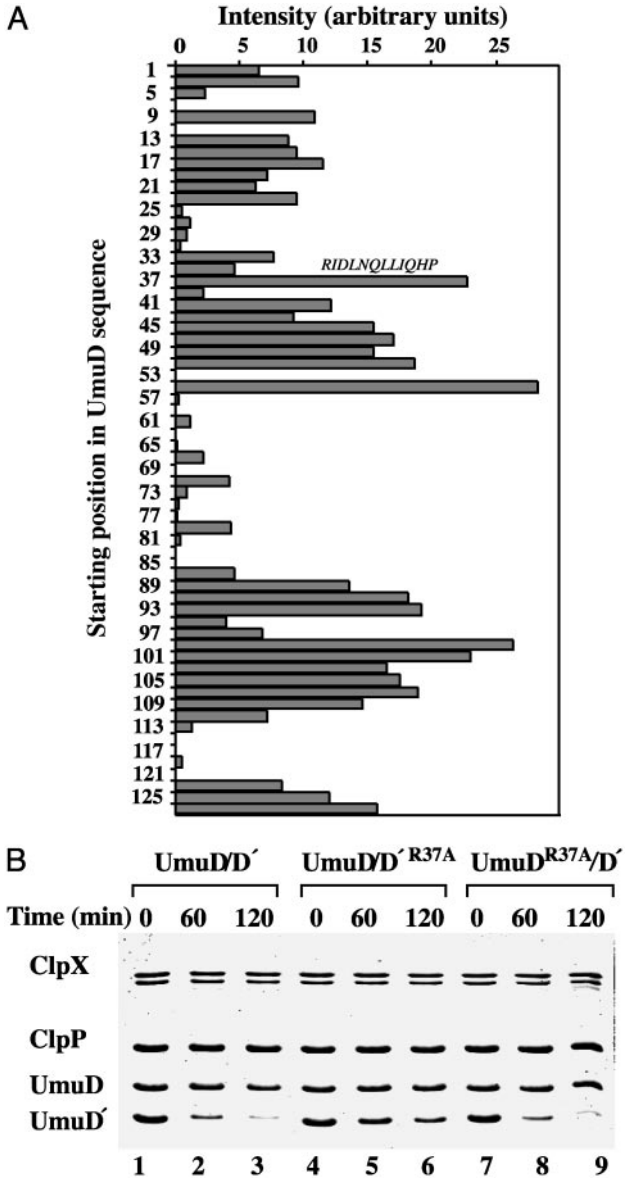


Fig. 5. (*A*) Overlapping 12-residue peptides from the UmuD sequence were arrayed by covalent attachment to a membrane, incubated with ClpX, and washed, and bound ClpX was detected by far-Western blotting using an anti-ClpX antibody and quantified by spot intensity. The sequence position of the N-terminal residue in the UmuD sequence for every other peptide is listed. (*B*) The R37A mutation reduces ClpXP degradation of the UmuD′ subunit of the heterodimer when it is present in the UmuD′ but not the UmuD subunit.

or ATP (Fig. 3*A*), indicating it is in fact due to the activity of the ClpXP enzyme.

To search for potential degradation signals in UmuD or UmuD′ that might interact with the substrate-processing site of ClpX, we probed a peptide array for sequences that bind ClpX. This array consisted of a set of 12-residue UmuD peptides covalently linked to a nitrocellulose filter, with each peptide sharing a 10-residue overlap with its neighbors. ClpX-interacting regions were identified in far-Western blotting by using ClpX and an anti-ClpX antibody. Three regions present in both UmuD and UmuD′ (residues ≈33–37, 41–51, and 85–109) interacted most strongly with ClpX (Fig. 5*A*). Based on the structure of UmuD′ (32), these sequences all contain residues exposed on the protein surface. None, however, showed strong similarity to other ClpX-targeting motifs that have been reported (9).

We tested the importance of the most N-terminal of these ClpX-binding regions by constructing variants of UmuD and UmuD′ with an R37A mutation (numbering relative to the UmuD sequence). This arginine was chosen for mutagenesis because positively charged amino acids seem to be important in ClpX recognition of many substrate degradation signals (9). When present in the UmuD′ subunit of a UmuD/D′ heterodimer, the R37A mutation caused this subunit to be degraded substantially more slowly that its wild-type counterpart (compare lanes 1–3 and 4–6 in Fig. 5*B*). In contrast, when the mutation was present on the UmuD subunit of a $UmuD^{R37A}$/D′ heterodimer, degradation of the UmuD′ subunit occurred as efficiently as with wild-type UmuD (lanes 7–9 in Fig. 4*B*). Control experiments demonstrated that both mutant proteins retained the ability to form dimers (data not shown). Thus, although the R37A mutation fails to completely block degradation of UmuD′, these experiments reveal that sequence information within UmuD′ can influence the efficiency of its recognition/degradation by ClpXP. These data support the idea that UmuD′ (and UmuD) contain one or more weak primary degradation signals that are recognized by the substrate-processing site on ClpX and are therefore important for ClpXP degradation.

Discussion

The results presented here support a model in which the UmuD/D′ complex must interact with ClpX at distinct tethering and substrate-processing sites for efficient ClpXP degradation to occur. UmuD carries a specific peptide motif that interacts with ClpX at the tethering site whereas its UmuD′ partner has one or more weak degradation signals recognized by the substrate-processing site. UmuD therefore functions in a manner analogous to SspB to deliver a bound protein partner to ClpXP for degradation. In fact, UmuD and SspB carry related sequence motifs (*LREI* in UmuD and *LRVV* in SspB) that are important for tethering to ClpX, and both occur within inherently flexible regions of each protein. In each case, these tethering interactions would enhance degradation by increasing the effective concentration of the degradation signal(s) on the partner molecule relative to the substrate-processing site of ClpX. Because the ClpX N-terminal domain is required for both SspB- and UmuD-mediated delivery, we assume that the tethering site is located within the N-terminal domain. The substrate-processing site, by contrast, must be part of the AAA+ core of ClpX because this portion of ClpX is fully active in the degradation of certain substrates (33).

It is important to note that delivery or trans-targeting for ClpXP degradation is not a general property of any oligomeric complex in which one subunit contains a ClpX degradation tag or a tethering motif. For example, ClpXP unfolds and degrades only the subunit(s) bearing a degradation tag in hetero-multimers containing tagged and untagged subunits (16, 34). Similarly, SspB binds to but fails to stimulate ClpXP degradation of a substrate in which the ClpX-interaction residues of the ssrA degradation tag have been mutated (18). These observations emphasize the dual requirement for a degradation signal and a tethering sequence for trans-targeting. Bipartite peptide signals required for ClpXP degradation have also been documented for σ^S and CtrA (19, 35), substrates for which delivery factors are known or suspected to be involved in ClpXP degradation. In these cases, one signal is likely to mediate interaction with the delivery factor and its tethering motif and the other with ClpX. In principle, a single protein could also interact with ClpX via a tethering motif and a degradation tag if these sequences were far enough apart and positioned in a way that allowed simultaneous contacts with their respective interaction sites in ClpX. This model, for example, could explain why the determinants of ClpXP degradation of the λ O protein are complex and involve multiple peptide sequences (8).

The experiments presented here demonstrate that the $UmuD_2$ and $UmuD'_2$ homodimers can be degraded by ClpXP. $UmuD_2$

homodimers are degraded by ClpXP with a K_m similar to that for degradation of UmuD/D′ heterodimers (Figs. 1*B* and 3*B*) whereas $UmuD'_2$ homodimers are much poorer substrates. By contrast, previous studies reported that the UmuD′ subunit of a UmuD/D′ heterodimer was the only form of the protein degraded by ClpXP, suggesting that ClpX recognition required a unique signal present only in the heterodimer (25). Our results support a different model: namely, that UmuD and UmuD′ contain low-affinity signals for ClpXP degradation, which are recognized efficiently only when the substrate is tethered to ClpX via a UmuD partner subunit. Peptide-binding studies and mutagenesis suggest that a sequence around Arg-37 in UmuD may serve as one such degradation signal.

Importantly, our results are in complete agreement with the previous conclusion of Woodgate and colleagues (25) that UmuD can catalytically target UmuD′ for degradation. Consistent with this model, we find that excess UmuD′ inhibits UmuD degradation. This result supports our model that one subunit of the dimer must be tethered to ClpX for the other subunit to be efficiently recognized and degraded. Because the *LREI*-tethering motif is absent from UmuD′, only UmuD can make the tethering interaction. With the additional assumption that a single subunit of UmuD cannot simultaneously contact the tethering and substrate processing sites on ClpX, this model explains why the UmuD′ molecule in the UmuD/D′ heterodimer is always the subunit degraded.

This hierarchy of UmuD and UmuD′ interactions with ClpX is undoubtedly important in regulating proteolysis in the cell. As noted previously (25), the trans-targeting of UmuD′ to ClpXP by UmuD provides a mechanism to limit UmuD′ availability and therefore to reduce error-prone DNA synthesis, which is catalyzed by a complex of UmuC with a $UmuD'_2$ homodimer (36, 37). Because UmuD/D′ heterodimers form preferentially (24), UmuD′ will be degraded by ClpXP whenever UmuD is also present at a concentration sufficient to support heterodimer formation, with proteolysis then releasing the UmuD subunit to target additional molecules of UmuD′ for destruction. As a consequence, $UmuD'_2$ homodimers will accumulate only when the vast majority of UmuD has been converted via DNA-damage/RecA-mediated autocleavage to UmuD′, that is, when DNA damage is at its worst and the need for repair is most urgent. ClpXP degradation of UmuD′ would be most important during the recovery phase after DNA damage, allowing existing UmuD′ subunits to be destroyed after damage had been repaired and autocleavage had stopped. It is unclear whether ClpXP degradation of $UmuD_2$ homodimers plays any significant intracellular role because Lon protease degrades these molecules efficiently (38).

In principle, tethering sites could occur at many positions on ClpX as long as the binding of the delivery protein did not prevent substrate binding and/or processing. In this regard, it is interesting that SspB and UmuD seem to use a common tethering site. Both delivery proteins contain similar tethering motifs and have a common need for the N-terminal domain of ClpX for these interactions. Moreover, the tethering motif of SspB substitutes for that of UmuD and blocks the UmuD interaction when added in trans as a peptide. The use by multiple delivery proteins of a common tethering site on ClpX could permit an additional layer of cellular regulation. By competition for this site, the synthesis of a new delivery factor in response to environmental cues could alter the "prioritization" of substrates for ClpXP degradation.

We thank Graham Walker for advice and enthusiastic support throughout this project, Samia Siddiqui for $ClpX^{\Delta 1-46}$, Elizabeth Courtenay for mass spectrometry, and Samia Siddiqui, Igor Levchenko, Greg Hersch, and David Wah for sharing results before publication. This work was supported by National Institutes of Health Grants AI-16892 and GM-499224 and the Howard Hughes Medical Institute.

1. Damerau, K. & St. John, A. (1993) *J. Bacteriol.* **175,** 53–63.
2. Weichart, D., Querfurth, N., Dreger, M. & Hengge-Aronis, R. (2003) *J. Bacteriol.* **185,** 115–125.
3. Gottesman, S., Clark, W. P., de Crecy-Lagard, V. & Maurizi, M. R. (1993) *J. Biol. Chem.* **268,** 22618–22626.
4. Maurizi, M. R., Clark, W. P., Katayama, Y., Rudikoff, S., Pumphrey, J., Bowers, B. & Gottesman, S. (1990) *J. Biol. Chem.* **265,** 12536–12545.
5. Kim, Y. I., Burton, R. E., Burton, B. M., Sauer, R. T. & Baker, T. A. (2000) *Mol. Cell* **5,** 639–648.
6. Singh, S. K., Grimaud, R., Hoskins, J. R., Wickner, S. & Maurizi, M. R. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 8898–8903.
7. Wang, J., Hartling, J. A. & Flanagan, J. M. (1997) *Cell* **91,** 447–456.
8. Gonciarz-Swiatek, M., Wawrzynow, A., Um, S. J., Learn, B. A., McMacken, R., Kelley, W. L., Georgopoulos, C., Sliekers, O. & Zylicz, M. (1999) *J. Biol. Chem.* **274,** 13999–4005.
9. Flynn, J. M., Neher, S. B., Kim, Y. I., Sauer, R. T. & Baker, T. A. (2003) *Mol. Cell* **11,** 671–683.
10. Gottesman, S., Roche, E., Zhou, Y. & Sauer, R. T. (1998) *Genes Dev.* **12,** 1338–1347.
11. Marshall-Batty, K. R. & Nakai, H. (2003) *J. Biol. Chem.* **278,** 1612–1617.
12. Neher, S. B., Flynn, J. M., Sauer, R. T. & Baker, T. A. (2003) *Genes Dev.* **17,** 1084–1089.
13. Kenniston, J. A., Baker, T. A., Fernandez, J. M. & Sauer, R. T. (2003) *Cell* **114,** 511–520.
14. Tu, G. F., Reid, G. E., Zhang, J. G., Moritz, R. L. & Simpson, R. J. (1995) *J. Biol. Chem.* **270,** 9322–9326.
15. Keiler, K. C., Waller, P. R. & Sauer, R. T. (1996) *Science* **271,** 990–993.
16. Burton, B. M., Williams, T. L. & Baker, T. A. (2001) *Mol. Cell* **8,** 449–454.
17. Zhou, Y., Gottesman, S., Hoskins, J. R., Maurizi, M. R. & Wickner, S. (2001) *Genes Dev.* **15,** 627–637.
18. Levchenko, I., Seidel, M., Sauer, R. T. & Baker, T. A. (2000) *Science* **289,** 2354–2356.
19. Studemann, A., Noirclerc-Savoye, M., Klauck, E., Becker, G., Schneider, D. & Hengge, R. (2003) *EMBO J.* **22,** 4111–4120.
20. Wah, D. A., Levchenko, I., Reickhof, G. E., Bolon, D. N., Baker, T. A. & Sauer, R. T. (2003) *Mol. Cell* **12,** 355–363.
21. Wah, D. A., Levchenko, I., Baker, T. A. & Sauer, R. T. (2002) *Chem. Biol.* **9,** 1237–1245.
22. Burckhardt, S. E., Woodgate, R., Scheuermann, R. H. & Echols, H. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1811–1815.
23. Shinagawa, H., Iwasaki, H., Kato, T. & Nakata, A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1806–1810.
24. Battista, J. R., Ohta, T., Nohmi, T., Sun, W. & Walker, G. C. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 7190–7194.
25. Gonzalez, M., Rasulova, F., Maurizi, M. R. & Woodgate, R. (2000) *EMBO J.* **19,** 5251–5258.
26. Dougan, D. A., Weber-Ban, E. & Burkau, B. (2003) *Mol. Cell* **12,** 373–380.
27. Wojtyra, U. A., Thibault, G., Tuite, A. & Houry, W. A. (2003) *J. Biol. Chem.*, 10.1074/jbc.M307825200.
28. Robinson, C. R. & Sauer, R. T. (1996) *Biochemistry* **35,** 13878–13884.
29. Sutton, M. D., Guzzo, A., Narumi, I., Costanzo, M., Altenbach, C., Ferentz, A. E., Hubbell, W. L. & Walker, G. C. (2002) *DNA Repair (Amsterdam)* **1,** 77–93.
30. Lee, M. H., Ohta, T. & Walker, G. C. (1994) *J. Bacteriol.* **176,** 4825–4837.
31. Ferentz, A. E., Opperman, T., Walker, G. C. & Wagner, G. (1997) *Nat. Struct. Biol.* **4,** 979–983.
32. Ferentz, A. E., Walker, G. C. & Wagner, G. (2001) *EMBO J.* **20,** 4287–4298.
33. Singh, S. K., Rozycki, J., Ortega, J., Ishikawa, T., Lo, J., Steven, A. C. & Maurizi, M. R. (2001) *J. Biol. Chem.* **276,** 29420–29429.
34. Burton, R. E., Siddiqui, S. M., Kim, Y. I., Baker, T. A. & Sauer, R. T. (2001) *EMBO J.* **20,** 3092–3100.
35. Ryan, K. R., Judd, E. M. & Shapiro, L. (2002) *J. Mol. Biol.* **324,** 443–455.
36. Tang, M., Shen, X., Frank, E. G., O'Donnell, M., Woodgate, R. & Goodman, M. F. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 8919–8924.
37. Tang, M., Bruck, I., Eritja, R., Turner, J., Frank, E. G., Woodgate, R., O'Donnell, M. & Goodman, M. F. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 9755–9760.
38. Gonzalez, M., Frank, E. G., Levine, A. S. & Woodgate, R. (1998) *Genes Dev.* **12,** 3889–3899.